Acta Derm Venereol (Stockh) 1997; 77: 428–431

Keratinocytes Cultured under Hyperthermal Conditions Secrete Factor(s) which Can Modulate Dermal Fibroblast Proliferation and Extracellular Matrix Production

N. FUJIMOTO, Y. ITOH, S. TAJIMA and A. ISHIBASHI

Department of Dermatology, National Defense Medical College, Saitama, Japan

We have studied how keratinocytes cultured under hyperthermal conditions modulate skin fibroblast growth potential and their biosynthetic phenotypes in vitro. When keratinocytes were cultured at 30, 34, 37 or 39°C, the conditioned medium of the keratinocytes cultured at 39°C showed a greater inhibitory activity for fibroblast proliferation and greater synthetic activities of collagen and glycosaminoglycans than those incubated at 30, 34, or 37°C. Transforming growth factor (TGF)β1 production in skin fibroblasts was also stimulated by the keratinocyte conditioned medium cultured at 39°C. The stimulating activity of collagen and glycosaminoglycan syntheses of keratinocyte conditioned medium may be explained at least partly by enhanced TGFβ1 production. The results indicate that keratinocytes cultured at a higher temperature (39°C) may secrete factor(s) which modulate both fibroblast growth and matrix synthesis. This may provide evidence that under hyperthermal conditions epidermis can influence the functions of skin fibroblasts. ***Key words: hyperthermia; collagen cell proliferation; glycosaminoglycan.***

(Accepted May 16, 1997.)

Acta Derm Venereol (Stockh) 1997; 77: 428–431.

N. Fujimoto, Department of Dermatology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359, Japan.

At physiological condition, the temperature of the epidermal layer, which faces the atmosphere and lacks circulating blood flow, is lower (25–30°C) than that of the dermis. Epidermal cells in culture have been shown to have optimal cell growth at 27–34°C (1), temperatures lower than those used in conventional cell culture systems (37°C). This implies that epidermal cell functions may differ depending on the culture temperatures.

Skin fibroblasts are thought to be responsible for the accumulation of extracellular matrices during wound healing. In the past, most wound care has consisted of providing an optimal environment for the wound. A good environment for cell growth and movement is created by warming or debriding the wound (2), controlling infection, and using pharmacological agents or different dressings (3). Hyperthermal therapy has been shown to improve a number of skin diseases, such as psoriasis (4), fungal infections (5, 6) and malignant skin tumors (7), and to improve delayed wound healing, possibly by increasing the blood flow of the dermal tissue, which results in the activation of skin fibroblast functions (8). However, it is also possible that some factors derived from epidermal cells may be induced by higher temperatures and modulate the proliferation and extracellular matrix production of dermal fibroblasts.

In the present report, we have attempted to study whether the conditioned media of keratinocytes cultured at different temperatures possess differential modulatory effects on mitogenic and biosynthetic phenotypes of dermal fibroblasts.

MATERIAL AND METHODS

Materials

Normal human keratinocytes (NHKs), originated from foreskin, were purchased from Sanko Junyaku (Tokyo, Japan). Highly purified bacterial collagenase isolated from *Cl. histolyticum* came from Advance Biofactures. Radioactive materials, [2,3-^{3}H]proline (2.2 TBq/mmol) and [6-^{3}H]glucosamine hydrochloride (1.2 TBq/mmol) came from Amersham.

Cell culture

NHKs were plated at a density of 2×10^4/35 mm-diameter-type I collagen-coated dish (collaborative Biomedical Products, Bedford, MA) and cultured for 3 days at 37°C in a medium containing insulin, epidermal growth factor (EGF), hydrocortisone and bovine pituitary extract (BPE) (designated as KGM) according to the supplier's protocols. Cultures were continued for 48 h at the temperatures of 30, 34, 37 and 39°C. At the termination of the cultures, the cell number was counted after trypsin (1:250) (Gibco) treatment, using a hemocytometer.

Normal human fibroblasts were established by a routine explant method. Fibroblasts were plated at a density of 1×10^5/35 mm dish and grown for 3 days at 37°C in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS); incubation temperatures were then shifted to 30, 34, 37 or 39°C. Cultures were continued for 48 h. At the end of the cultures, the cell number was counted as described above.

To study the effects of the conditioned medium of keratinocytes on fibroblast proliferation, NHKs were cultured for 3 days at 37°C in a medium containing insulin, EGF, hydrocortisone and BPE. The medium was replaced with serum-free DMEM to exclude the influences of the factors contained in the keratinocyte medium, and incubation temperatures were shifted to 30, 34, 37 or 39°C. Cultures were continued for 48 h. At the termination of the incubation, the medium was harvested and stored at −80°C until ready for use. Fibroblast cultures on day 3 after inoculation received the conditioned medium at 37°C for 48 h, and the cell number was then counted.

In one experiment, the conditioned medium harvested from keratinocytes was heat-denatured at 100°C for 2 min prior to the treatment of fibroblasts.

Metabolic labeling and determination of collagen synthesis in skin fibroblasts

Fibroblast cultures were treated with keratinocyte-conditioned medium for 2 days and labeled with [2,3-^{3}H]proline (20 μCi/ml) in the presence of ascorbic acid (30 μg/ml) during the last 24 h of the incubation. The medium was then collected and mixed with protease inhibitor cocktails, to yield 1 mM each of N-ethylmaleimide, EDTA (pH 7.0) and phenylmethylsulfonyl fluoride. The cells were harvested with 0.25% trypsin, and the cell number was determined with a hemocytometer. After centrifugation cells were combined with the medium and stored at −20°C until ready for analysis. The amount of radioactivity incorporated into collagen was determined using

© 1997 Scandinavian University Press. ISSN 0001-5555

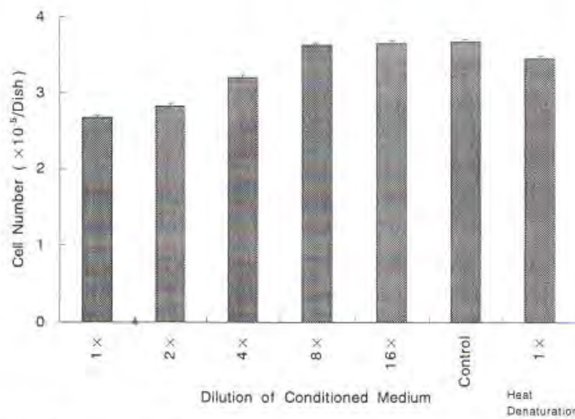

Fig. 1. Normal human keratinocytes were cultured at 39°C for 48 h in serum-free DMEM. The medium was serially diluted to 16× with DMEM and placed on fibroblasts, as described. Control indicates DMEM alone, which had been placed in the dishes at 39°C for 48 h. The cell number was counted with a hemocytometer. Values are means±SD from triplicate experiments.

purified bacterial collagenase (9), as previously described (10). Synthesis of collagen was expressed as collagenase-sensitive cpm/cell.

Synthesis of glycosaminoglycans in cultured skin fibroblasts

Fibroblasts were treated for 48 h with keratinocyte culture media and labeled with [6-^{3}H]glucosamine (25 μCi/ml) for the last 24 h of the treatment. Cultured medium and cell pellet following trypsin treatment were combined together with carrier glycosaminoglycan mixtures, consisting of hyaluronic acid, dermatan sulfate, chondroitin 4- and 6-sulfate and heparan sulfate, and were then lyophilized. Isolated glycosaminoglycans were subjected to two-dimensional electrophoresis on cellulose-acetate membranes, using 0.1 M pyridine-0.45 M formic acid, pH 3, at 1 mA/cm for 1.5 h in the first dimension and 0.1M barium acetate, pH 8, at 1 mA/cm for 5 h in the second. The radioactivities incorporated in total glycosaminoglycan or in each component were determined with a liquid scintillation spectrometer (Hitachi 124) (11–13).

Determination of transforming growth factor (TGF)-β1 in cultured skin fibroblasts

TGF-β1 levels in the medium were determined by the ELISA system, using monoclonal antibody for TGFβ1 (Amersham). Quantitative determination was performed within the linear range between input sample amount (0–1000 pg/ml TGFβ1) and optical absorbance at 492 nm, according to the supplier's protocol. Values are expressed as pg per cell number.

RESULTS

As keratinocyte culture medium contains several growth promoting factors (insulin, EGF, hydrocortisone and BPE) in a conventional culture system, we utilized DME medium for conditioned medium to properly address the effect of keratinocyte-derived factor(s) on fibroblast proliferation. No cellular toxicity was found in the keratinocytes cultured at lower (30°C) or higher (39°C) temperatures for 2 days in DMEM, on the basis of the trypan blue exclusion test (not shown) and unaltered keratinocyte proliferation (see below).

When keratinocytes and fibroblasts were cultured at 30, 34, 37 and 39°C for 2 days, there was no significant difference in mitotic activity in either cells ($11.5–12.1 \cdot 10^4$ and $2.4–2.5 \cdot 10^5$ per dish, respectively). This result tempted us to explore the possibility whether the keratinocyte culture medium may potentially modulate fibroblast proliferation.

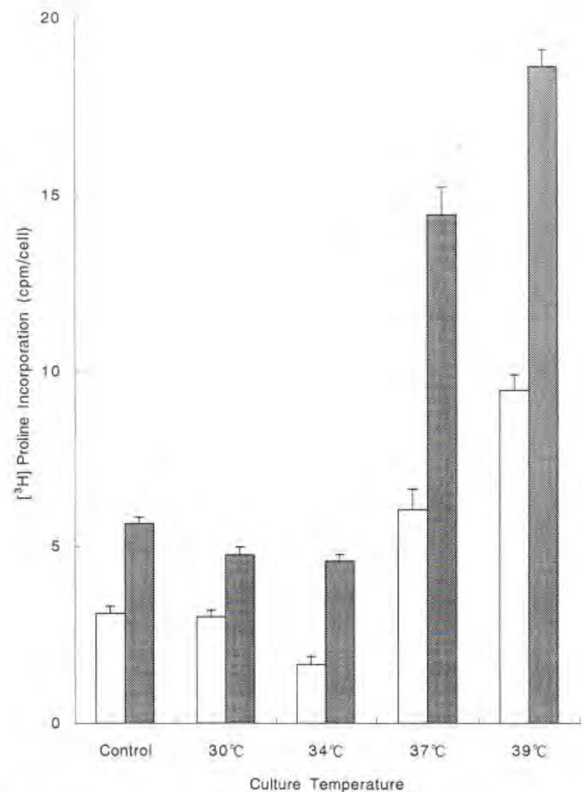

Fig. 2. Effects of keratinocyte culture temperatures on collagen synthesis in skin fibroblasts. Keratinocyte media cultured at 30, 34, 37 or 39°C were placed on fibroblast cultures and incubated for 2 days and were then labeled with [^{3}H]proline for the final 24 h of the treatment in the presence of ascorbic acid (30 μg/ml). Control experiments were performed with the media alone. Collagen synthesis was assayed with bacterial collagenase digestion. Collagen synthesis (*open columns*) and total protein synthesis (*closed columns*) were expressed by radioactivity/cell number. Values are means±SD from triplicate experiments.

Keratinocyte medium cultured at 30, 34 and 37°C did not exhibit any significant effects on fibroblast proliferation. By contrast, the medium harvested from the culture at 39°C did inhibit fibroblast proliferation in one third of the controls in triplicate experiments.

With the serial dilution of conditioned medium prepared from the keratinocytes cultured at 39°C, the inhibitory effect on fibroblast proliferation was reduced to the control level. When the conditioned medium was heated at 100°C for 2 min, the inhibitory effect was abolished (Fig. 1).

Collagen and total protein syntheses of fibroblasts were unchanged with the treatments of keratinocyte-conditioned medium cultured at 30 and 34°C. The conditioned medium cultured at 37°C or 39°C slightly or significantly stimulated both collagen and total protein syntheses (Fig. 2).

Culture temperature-dependent stimulation of total glycosaminoglycan synthesis in skin fibroblasts was observed by keratinocyte-conditioned medium (Fig. 3). The major components of glycosaminoglycan synthesized by skin fibroblasts were found to be hyaluronic acid and dermatan sulfate. There were no significant differences in the relative synthesis of the two glycosaminoglycans (data not shown).

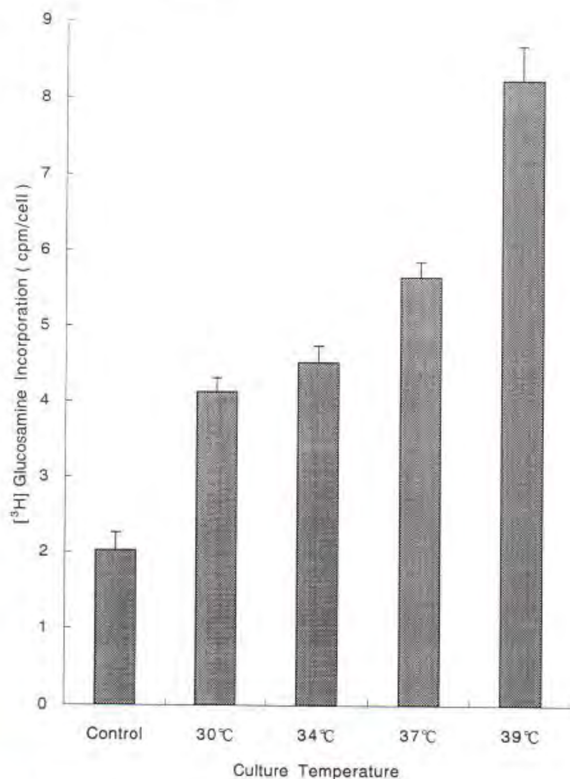

Fig. 3. Effects of keratinocyte-conditioned medium cultured at various temperatures on glycosaminoglycan synthesis by skin fibroblasts. Keratinocytes were cultured at 30, 34, 37 and 39°C for 48 h in serum-free DMEM. The medium was harvested and transferred to cultured fibroblast culture, continued for 2 days, and labeled with [^{3}H]glucosamine for the final 24 h of treatment. Control experiments were performed with the media alone. Crude glycosaminoglycans were extracted from medium and cells. An aliquot was taken and counted for determining total incorporation of [^{3}H]glucosamine. Values are means ± SD from duplicate experiments.

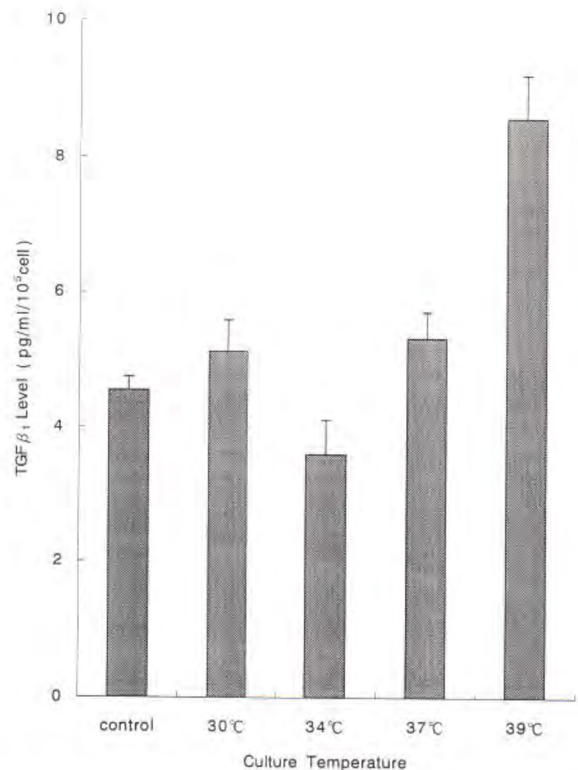

Fig. 4. Effects of keratinocyte-conditioned medium cultured at various temperatures on TGFβ1 production of skin fibroblasts. Keratinocytes were cultured at 30, 34, 37 and 39°C for 48 h in serum-free DMEM. The medium was harvested and transferred to cultured fibroblasts. Fibroblasts were cultured for 2 days. The medium was harvested, and the TGFβ1 content in the medium was determined with ELISA assays on the basis of a calibration curve with various amounts of authentic TGFβ1. Values are normalized by cell number and expressed as means ± SD from duplicate experiments.

The TGFβ1 level in the fibroblast cultures was elevated by the keratinocyte-conditioned medium cultured at 39°C, whereas keratinocyte medium cultured below 37°C did not influence the TGFβ1 level, compared with the controls (Fig. 4).

DISCUSSION

Cultured keratinocytes have been demonstrated to produce a number of factors affecting dermal fibroblast phenotypes. These include EGF, interleukin (IL)-1, tumor necrosis factor (TNF), TGF-β, interferon (IFN)-γ, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) (14). Out of these, only TGFβ and PDGF are known to possess the stimulating activity of extracellular matrix production in skin fibroblasts, but these two growth factors do not exhibit inhibitory effects on fibroblast proliferation (15, 16). This suggests that hyperthermia-induced keratinocyte factor(s) may be unique or comprise more than two growth factors.

It is noted that the conditioned media of keratinocyte cultured at 39°C stimulated the production of TGFβ1. Since TGFβ1 is known to be a potent stimulator of collagen and proteoglycan syntheses (17–19) and is considered to play a key role in the accumulation of connective tissues in scleroderma, keloid and wound healing, it may be postulated that the stimulation of matrix protein synthesis by keratinocyte-conditioned media is mediated at least in part by the stimulation of TGFβ1 production.

Hyperthermia has been utilized in the treatment of human malignancies. Hyperthermia is known to show a direct cytotoxic effect at temperatures above 41°C and to be selectively lethal to cancer cells (20). Our results show that hyperthermal treatment of skin at 39°C can alter the fibroblast function via a soluble factor derived from epidermis. This may provide evidence that skin fibroblasts undergo a critical influence from epidermis during wound healing and fibrotic conditions, such as keloid and scleroderma. Hyperthermal conditions may serve as an accerelator of delayed wound healing.

REFERENCES

1. Indo K, Wilson RB. Fetal rat keratinizing epidermal cells in culture: effects of long-term treatment by benzo[a]pyrene on their growth characteristics. J Natl Cancer Inst 1977; 59: 867–880.
2. Meltzer T, Myers B. The effect of hyperbaric oxygen on the bursting strength and rate of vascularization of skin wounds in the rat. Am Surg 1986; 52: 659–662.
3. Kaufman T, Nathan P, Levin M. Drug-loaded synthetic dressing: effect on contraction, epithelialization, and collagen synthesis of deep second-degree experimental burns. Ann Plastic Surg 1985; 14: 420–427.

4. Urabe H, Nishitani K, Kohda H. Hyperthermia in the treatment of psoriasis. Arch Dermatol 1981; 117: 770–774.
5. Tagami H, Ginoza M, Imaizumi S. Successful treatment of chromoblastomycosis with topical heat therapy. J Am Acad Dermatol 1984; 10: 615–619.
6. Hiruma M, Kato T, Yamamoto I, Kagawa S. Local hyperthermia in the treatment of sporotrichosis. Mykosen 1987; 30: 315–321.
7. Draelos ZK, Levine N. Hyperthermic treatment of cutaneous malignancies. J Am Acad Dermatol 1983; 9: 623–628.
8. Song CW, Kang MS, Rhee JG, Levitt SH. Effect of hyperthermia on vascular function in normal and neoplastic tissues. Am NY Acad Sci 1980; 335: 35–47.
9. Peterkofsky B, Diegelmann R. Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry 1971; 10: 988–994.
10. Phillip CL, Tajima S, Pinnell SR. Ascorbic acid and transforming growth factor-β1 increase collagen biosynthesis via different mechanisms: coordinate regulation of pro α1(I) and pro α1(III) collagens. Arch Biochem Biophys 1992; 295: 397–403.
11. Hata R, Nagai Y. A rapid and micro method for separation of acidic glycosaminoglycans by two-dimensional electrophoresis. Anal Biochem 1972; 45: 462–468.
12. Hata R, Nagai Y. A micro colorimetric determination of acidic glycosaminoglycans by two-dimensional electrophoresis on a cellulose acetate strip. Anal Biochem 1973; 52: 652–656.
13. Ninomiya Y, Hata R, Nagai Y. Glycosaminoglycan synthesis by liver parenchymal cell clones and its change with transformation. 1962; 675: 248–255.
14. Nickoloff BJ. Cytokine networks in skin disease. In: Kunkel SL, et al., eds. Cytokines in health and disease. New York: Marcel Dekker, 1992: 413–432.
15. Lyons RM, Moses HL. Transforming growth factors and regulation of cell proliferation. Eur J Biochem 1990; 187: 467–473.
16. Seppä H, Grotendorst G, Seppä S, Schiffmann E, Martin GR. Platelet-derived growth factor is chemotactic for fibroblasts. J Cell Biol 1982; 92: 584–588.
17. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, et al. Transforming growth factor β: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 1986; 83: 4167–4170.
18. Ignotz RA, Massague J. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 1986; 261: 4337–4345.
19. Falanga V, Tiegs SL, Abstadt SP, Roberts AB, Sporn MB. Transforming growth factor β: selective increase in glycosaminoglycan synthesis by cultures of fibroblasts from patients with progressive systemic sclerosis. J Invest Dermatol 1987; 89: 100–106.
20. Manning MR, Cetas TC, Miller RC, Oleson JR, Connor WG, Gerner EW. Clinical hyperthermia: results of a phase I trial employing hyperthermia alone or in combination with external beam or interstitial radiotherapy. Cancer 1982; 49: 205–216.